# IMPROVED TECHNIC FOR THE MICRO OR LITTLE PLATE METHOD OF COUNTING BACTERIA IN MILK

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## INTRODUCTION

I have previously described a method for counting bacteria in milk and other richly seed material.[1] It is possible by means of this method to get counts in a few hours. It has been found also, by myself [2] and Simmons,[3] that the count obtained is reasonably close to that given by the Koch plate method.

In the last few years the method has been put to practical test and, furthermore, since the technic has been improved in several important particulars, it seems necessary to give a description of the method in detail.

When it was first published the impression was conveyed to some, at least, that it would be necessary to limit the period of incubation to a few hours and this was interpreted as a disadvantage because of the necessity of coming to the laboratory "out of hours" in order to take the plates out of the incubator and dry them. In reality, however, the incubation period can be extended to 16 or even 24 hours without affecting the count. It is thus possible to make the cultures one day and leave them in the incubator until a convenient time the next day.

By using a smaller pipet, it is possible to measure the sample directly onto the slide; this shortens the method, obviates the necessity of diluting the sample, economizes the medium and materially simplifies the method.

The special apparatus needed has been standardized and satisfactory types put in commercial form. A portable outfit, in a combination

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[1] Jour. Am. Med. Assn., 1916, 66, p. 889.
[2] Jour. Infect. Dis., 1916, p. 237; Jour. Bacteriol., 1917, 2, p. 567.
[3] Jour. Infect. Dis., 1919, 24, p. 322.

carrying case and incubator, has been devised, which ought to be of real value in the solution of field problems.

## APPARATUS NEEDED

*Glass Slides.*—The apparatus needed consists, first of all, of glass slides. These must have marked off on them a definite area, of 4 square cm. (fig 1). It would seem best to have such areas permanently fixed, and slides can be purchased so marked.[4] Since these are quite expensive, most workers will no doubt continue to use ordinary microscopic glass slides (1 by 3 inches) marked with a wax pencil. This grease border line has a distinct advantage since it causes the milk and agar to flow back and so the marked off areas are not overrun in making the "little plates." In order to have the areas accurate, it is best to use some mechanical means for the purpose of marking them. The apparatus illustrated in fig. 2 is suggested as satisfactory, although it is possible to mark off the areas fairly accurately by tracing the lines over a paper pattern. It is economical to mark off two areas on each slide.

*Forceps.*—For handling the slides a good forceps is needed. Fig. 3 represents a desired form.

*Warm Table.*—In order to prevent the hardening of the agar before it is thoroughly mixed with the milk, it is necessary to spread the little plates on a "warm table." A convenient form is a metal box surrounded with asbestos on all sides except the top and bottom. In it warm water (45 C.) is placed. At one end, tubulations to hold two test tubes of medium are inserted at an angle of about 45 degrees (fig. 4).

*Special Pipets.*—One pipet about 8 in. long made of capillary tubing is suggested. The tube should be sufficiently small so that about one-half inch will hold one hundredth of a c c and a twentieth of a c c would equal about 2½ in. If the pipet is marked in 0.01 c c, the same pipet can be used to make a direct count (Breed), as well as the little plate count (fig. 5). Such pipets are now stocked by most apparatus firms as "serological pipets."

*Moist Chamber Cabinet.*—This is shown in fig. 6. It is simply a rack to hold 48 slides and is provided with a space for water in the bottom and room on the sides for the moisture to circulate. The rack is made removable. This makes it possible to prepare all of the slides for microscopic examination without handling them individually.

*Hot Plate or Drying Oven.*—It is necessary to dry the little plates rapidly, and this is best done by keeping them for about 5 minutes just below the boiling point of water. When the plates are handled individually, a metal box is used with a flat and level top in which water is kept at or near the boiling point. Where the slides are to be dried in the rack, an oven is necessary and an electric one is very satisfactory.

*Staining Outfit.*—For the purpose of staining the individual slides a staining jar is needed. A good form is the Coplin jar. A tumbler or larger jar is needed for the wash water. In case the whole rack of slides is to be stained at once a special container for the rack is needed.

[4] Central Scientific Co., Chicago, Ill.

## MATERIAL NEEDED

*Culture Medium.*—Ordinary nutrient agar is used. It is made and sterilized in the usual way (standard methods Am. P. H. Assn.). One test tube of medium (5 c c) will be sufficient for about 50 samples.

*Acetic Acid Solution.*—A 10% solution of glacial acetic acid in 95% alcohol.

*Stains.* — Methylene blue and thionine are both satisfactory. Loeffler's methylene blue diluted with three times its volume of distilled water has been used most.

It is also possible to make a satisfactory stain by taking 10 c c of a saturated alcohol solution of methylene blue to 400 c c of distilled water.

The formula for the thionine stain is: thionine blue, 1 gm.; carbolic acid, 2½ gm., and distilled water, 400 c c; filter. To this is added 5% of glacial acetic acid. The slides are put in this stain without preliminary treatment with acid alcohol. We have found this the most satisfactory stain.

## METHOD IN DETAIL

*Melting Agar.*—A tube of agar is melted by placing it in boiling water. It is then placed in one of the tubulations on the warm table. The cotton plug is removed and a pipet which will deliver a small drop (about 0.05 c c) is placed in it.

*Filling the Warm Table.*—While the agar is melting, the warm table should be filled with water that has a temperature of about 45 C. It is necessary to note the temperature occasionally. If it gets down to 40 C., the agar is likely to harden, and if it gets much above 45 C., it is probable that some of the milk bacteria will be killed or at least devitalized to such an extent that they will not form colonies within the usual period of incubation.

*Mixing the Milk.*—The bottle or sample should be shaken at least 25 times by tipping it from end to end slowly enough to avoid the formation of a foam, since air bubbles would interfere with the accurate measurement of the sample in the pipet.

*Sterilizing the Glass Slides.*—The glass slides, which have been properly marked, are sterilized in the direct flame. In order to do this, they are taken up in the forceps and passed through the flame, about three times, marked surface down, and once on the other side. About a half dozen are thus sterilized and placed on the warm table.

*Pipetting the Sample.*—A special pipet has already been described. Approximate results can be obtained with any pipet that will measure a small quantity, as 0.05 c c, but since the accuracy of the count depends on the correct measurement of the sample, the construction and use of the pipet becomes a matter of great importance. It is necessary to use a pipet of small bore in order to measure accurately. This necessitates the use of heavy walled tubing. The end must be pointed, otherwise too much milk clings to the outside.

It is important, in using a pipet, not to dip it into the milk any farther than is necessary to avoid taking in air bubbles. The reason for this is that milk clings to the outside of the pipet and will run down and swell the sample. In any case, the tip of the pipet after it is filled should be brought in contact with the inside of the bottle above the milk to drain off the extra milk, or wiped with a piece of sterile filter paper or a sterile towel. Five one-

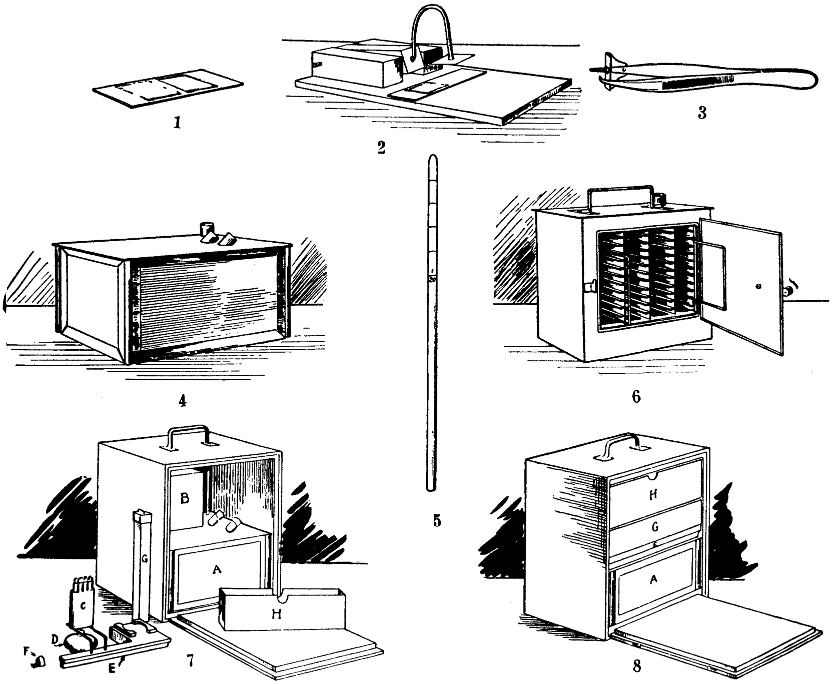


Fig. 1.—Microscopic glass slide with two areas of 4 sq. cm. each marked off with a wax pencil.

Fig. 2.—A guide for marking the slides.

Fig. 3.—Forceps provided with a stop which makes it possible to handle slides easily.

Fig. 4.—A "warm table." This is an asbestos covered metal box which holds about 2 liters of water and is used to keep the slides warm while the milk and agar are being spread to form the "little plates." It also serves to keep the tube of liquefied agar from solidifying. It has 3 tabulations, 2 at an angle for holding tubes of medium and the other for a thermometer.

Fig. 5.—A serologic pipet which delivers 0.01 c c; 0.05 is the amount of milk usually used.

Fig. 6.—A "moist chamber cabinet." The "little plates" are kept in this while they are in the incubator. Water in the bottom keeps a moist atmosphere. The rack is made removable so that all of the slides can be dried (in an oven), fixed and stained altogether, and thus avoiding the handling of each slide separately.

Fig. 7.—Field Outfit. The case is wood and is lined with an insolating material and when closed forms an incubator on the principle of a "fireless cooker." A is the "warm table" (fig. 4) which is filled with water 42-43 C. B is the "moist Chamber cabinet" (fig. 6) and contains the little plates. C is a cup which holds the agar tubes and in which they may be melted over the alcohol lamp D. E is a support for the cup and also holds the various articles in place when the case is packed. F is the cap to the alcohol lamp. G is the pipet case and H is a box in which are placed the glass slides, platinum loops, etc.

Fig. 8.—Carrying case packed ready to be closed. It then becomes an incubator. The outfit holds all the material necessary for making an analysis of 48 samples in duplicate.

hundredths ($\frac{1}{20}$) c c of the sample of milk is placed in each area marked off on the glass slides, i. e., two equal portions from each sample are put in different squares on the same slide.

The milk portions should be put on only a few slides at once because they begin to dry before the agar is added to them, if allowed to stand on the warm table.

*Adding Agar and Spreading Film.*—As soon as possible after the milk has been put on the glass slides, a drop of agar should be added to each. An exact amount is not absolutely demanded, but it should be approximately 0.05 c c, and pipets should be selected that will deliver that amount in one or two drops. Practically the same amount should be used each time, otherwise the concentration of the agar will vary, and consequently, its consistency. This variation does not appear to affect the number of the colonies, but it influences their appearance.

The milk and agar must be thoroughly mixed and spread evenly over the 4 sq. cm. area. This is exactly done by means of a sterile platinum loop.

In order to spread the films as quickly as possible, it is desirable to have two loops and some arrangement for holding them in a flame so that one can be sterilizing while the other is in use. If the slides are properly heated at the time of sterilization, the film is easily spread and can be made to cover evenly the entire area. When the film is properly spread, it must be hardened. If the shelves in the moist chamber cabinet hold the slides in a level position, they can be put directly there, otherwise it will be necessary to arrange a place to harden them. If the work table is level, they can be placed there under a cover which will protect them from air contamination. When the film is hard, they are ready for the moist chamber and incubation.

*Moist Chamber Cabinet.*—It is necessary to keep the little plates from drying down during incubation. For a few slides and as a makeshift Petri dishes can be used. In this case the Petri dish has the bottom covered with wet filter paper; on this are laid two glass rods or match sticks, and on these the slides may be placed, two to a dish. It is more convenient, however, to have the special apparatus designed for the purpose and called a "moist chamber cabinet," which has been described. When a considerable number of milk samples are to be examined, the cabinet is essential. It should be thoroughly cleaned from time to time to prevent contamination by such organisms as produce spreaders on ordinary plates. It is quite sufficient to wash the cabinet in hot water or boil a little water in the bottom of it by placing it over a flame. It should, of course, always be cooled before use, and when in use it should always have at least enough water in it to cover the bottom in order that the air may be kept moist.

*Incubation.*—The little plates are best incubated at a temperature of 37.5 C. The moist chamber cabinet is put in an ordinary incubator, although a heavily insulated case, together with a source of heat, similar in principle to a fireless cooker, can be used. The possibilities of this are discussed later.

The period of incubation varies. If it is necessary to get results at the earliest possible moment, even so short a period of incubation as 4 hours will be satisfactory if the bacteria are actively growing in the milk at the time of sampling. If the milk has come from cold storage, has been pasteurized or is fresh and low in bacteria, 7 or 8 hours are necessary; if, on the other hand,

there is no particular hurry about the results, the plates can be left in the incubator from one day to the next, i. e., 15 or 16 hours.

They can also be incubated at room temperature (20 C.). In this case it seems necessary to leave them for at least 24 hours. Even then the count seems lower than that obtained by incubating at 37 C., although this has not been fully established.

*Drying Films.*—When the little plates are taken from the incubator, they should be dried at once. If they are allowed to dry at the room or incubator temperature, the films have a tendency to crack and to peel off or loosen in the staining process. It is best to dry the films down rapidly by putting them on a hot plate or in a drying oven at a temperature slightly below that of boiling water.

Recent trials indicate that it is possible also to put the wet films directly in the acetic acid alcohol or even the stain when it contains the acetic acid.

*Staining.*—It is possible to stain the films so that the colonies are deeply colored, while the medium is almost wholly without color. The success of this process depends to a considerable extent on the treatment that the films receive before they are put in the stain. They must be properly dried as already indicated. They are then put in the acetic acid solution. This acts quickly but does not overact; hence, it should be applied for about one minute, but no harm is done if the exposure is a long one. The purpose of the acetic acid is to keep the agar from taking the stain. Any of the stains mentioned are satisfactory, and the time of exposure is about 2 minutes, although a longer exposure is not harmful. The thionine gives excellent results. The background is clearer than with methylene blue.

The slides should be thoroughly washed in tap water. When stained, the preparation should be dried, and for this purpose a drying plate is convenient; a temperature just below 100 C. is best.

*Dilution of Heavily Contaminated Samples.*—If a milk is likely to have more than one million bacteria per c c, it should be diluted before a little plate is made. When water blanks are used, as is the common practice with the Koch plate method, comparatively little milk is taken over into the culture. If the same method were followed with the little plates, the food conditions would be quite different in the direct plates and those diluted with water. In order, therefore, to have comparable conditions in all of the little plates, it is advised that sterile milk be used for dilution purposes instead of water. It is necessary to have tubes of sterile milk containing 9 c c. To these is added 1 c c of the milk to be counted, and then after thorough mixing the diluted sample is used in exactly the same way as the ordinary samples.

A simpler way, however, is recommended, especially for field work. This was suggested by W. D. Dotterer of the Bowman Dairy Co. A small fraction of a c c of the milk to be analyzed is used, 0.01 or 0.02 c c and to this, on the slide, a drop of sterile milk is added from a tube which is kept in the second tubulation on the warm table. To this is added the agar, and the whole is spread with perhaps more than usual care.

*Counting Colonies.*—The counting is done under a compound microscope. If the colonies are large and few in number, the low power can be used, and this is best. If the colonies are small or numerous, the higher powers must be used. With the immersion objective, the oil is put on the dried agar. There

is no objection to mounting them in balsam and using a cover glass. This is best if the high dry powers are to be used. At first it seemed necessary to count at least 20 fields widely distributed over the film in order to get a fair average. For this a mechanical stage was recommended. Further experience leads to the conclusion that, ordinarily at least, it is only necessary to count 5 fields. In this case the slide is put under the microscope and moved about to get a general idea of the distribution and then 5 representative fields are counted. In counting, several things should be kept in mind. First, all of the colonies within the fields should be counted and only half of those that touch the edge, say those on the right half of the periphery, while those touching the left edge of the field should not be counted. Second, with the highest powers single bacteria or small groups should not be counted, on those plates that have been incubated 7 or 8 hours or more. In plates incubated as long as that, practically all of the original live bacteria have grown into definite colonies. Here as elsewhere, experience and good judgment are necessary to get consistent results.

*Calculating the Number of Bacteria per Cubic Centimeter of Milk.*—Having determined the number of colonies in a given number of fields of the microscope, or the number in an average field, it is necessary to multiply that number by some factor which will convert the figures representing the number of colonies per field of the microscope into figures which will represent the total number of bacteria per c c of the milk.

In order to do this it is only necessary to determine the area of the microscopic field, for the value of all of the other factors is known, i. e., the area of the little plates (4 sq. cm.) and the amount of milk used (0.05 c c, 0.005 c c, etc.).

The following formula is used:

$$\left.\begin{array}{c}\text{The number of bacteria}\\ \text{per c c of milk}\end{array}\right\} = \frac{\text{No. colonies counted}}{\text{No. fields counted}} \times$$

$$\left.\frac{\text{Area of little plates}}{\text{Area of the microscopic field}}\right\} \text{(or microscopic factor)} \times$$

Reciprocal of the dilution of the milk.

Let:

Number of bacteria per c c of milk $=$ X

Number of colonies counted divided by the number of fields counted, i. e., the average number of bacteria in a microscopic field $=$ C.

The area of the little plates (4 sq. cm. or 200 sq. mm.), divided by the area of the microscopic field or microscopic factor $=$ M. The reciprocal of the dilution of the milk $=$ D.

Then, $X = C \times M \times D$. The only unknown value in the second half of the equation is the area of the microscopic field. This must be determined not only for each microscope, but also for each combination of lenses.

The area of a disk equals the radius squared times 3.14159 ($\pi$), or the diameter squared times 0.7854 ($\pi/4$).

In order to determine the diameter of the field of a microscope a stage micrometer is necessary. This should be ruled to 0.1 and 0.01 mm.

By regulating the length of the tube, the size of the field may be varied. It would seem desirable, however, to use the ordinary tube length, otherwise

there is always danger that the tube may not be properly drawn out and the count thus affected.

For the convenience of those who may not have a stage micrometer at hand, the following table is included, which gives the approximate diameters and the areas of the microscopic fields of an American microscope. This will give a value which will serve until there is an opportunity to have the diameter of the particular microscope carefully determined.

DIAMETER OF MICROSCOPE FIELDS WITH COMMON COMBINATION OF LENSES AND THE TUBE PUSHED IN (160) MM.

| Objective (Equivalent Focus) | Ocular | Diameter of Field | Area of Field |
|---|---|---|---|
| ⅔ inch or 16 mm............ | 10 x | 1.55 mm. | approximately 2 sq. mm. |
| ⅙ inch or 4 mm............ | 10 x | 0.31 mm. | approximately 0.08 sq. mm. |
| 1/12 inch or 2 mm............ | 10 x | 1.555 mm. | approximately 0.02 sq. mm. |

In order to make correction for eyepieces of different magnifying power, multiply the area given above for the 10 x ocular by 1.4, for an 8 x by 1.5, for a 7.5 x, by 2 for a 5 x and 2.5 for a 4 x. With a 16 mm. objective and a 10 x ocular the area of the microscopic field is approximately 2 sq. mm. In order to get the microscopic factor, the area of the little plate (400 sq. mm.) is divided by the area of the microscopic field (2 sq. mm.). This gives 200. This figure multiplied by the denominator of the fraction of a c c of the milk used in making the little plate gives the factor necessary to convert the colonies in the field of the microscope into bacteria per c c of milk. The usual dilution is 1/20 c c; this means that each colony in the field of the microscope represents 4,000 bacteria per c c.

The same figuring would show us that each colony under a 4 mm. objective would mean 100,000 bacteria per c c of milk and one colony under the oil immersion would mean 400,000 bacteria per c c of milk.

## FIELD OUTFIT

Because of the small amount of material required to make the cultures and its simplicity, this method ought to be of special value in field work.

A carrying case is here described which may also serve as an incubator.

It is shown packed in fig. 8 and its contents are exhibited in fig. 7. It is a case about 13¾ inches long, 13½ inches high and 9¾ inches wide. It is lined with an insulating material. In the particular case figured, the insulating material was made of flax straw. The important dimensions are the inside ones, 10¾ inches long, 11 inches high and 6⅛ inches wide. The door is arranged to close tightly; this is accomplished by using a thick felt strip in the jam. In this case are fitted the warm table A; the moist chamber cabinet B; a support for holding the needles and a cup for melting the agar over a flame, E; an alcohol lamp D; a cup for holding the medium and melting it C; a pipet case G; and a box for such articles as glass slides, needles, forceps, wax pencil, etc. The case is small and packed with material necessary for duplicate plates of 48 different samples. The weight is only about twenty pounds.

If one plate only were made, 96 samples could be analyzed. If the trip were to be long, a large number of samples could be analyzed by taking

extra slides, pipets and medium, provided one set of slides were incubated and dried down before the next set of plates were made. The dried plates keep indefinitely, either stained or unstained, in slide boxes.

To use the carrying case as an incubator, it is only necessary to fill the warm table with water at about 43 C., pack and close the case. When the case is kept at ordinary room temperature, the heat of the water will bring the chamber up to about that of blood heat and hold it above 30 C. for at least 12 hours. In this case the colonies grow out satisfactorily in from 8 to 16 hours.